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In Vitro Assessment of Cytotoxicity and Estrogenicity of Vivera® Retainers

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Abstract: AIM To investigate the cytotoxicity and estrogenicity of Vivera® retainers by assessing their biological behavioral effects as-received from the manufacturer and after retrieved from patients. **MATERIALS AND METHODS** In this, in vitro investigation six sets (maxillary and mandibular) of Vivera® retainers, three as received and three retrieved after four weeks of use by patients of an orthodontic postgraduate clinic, were immersed in the normal saline solution for 14 days following different modes of sterilization. The estrogenicity assays involved two cell lines, namely the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB-231. Following a 6 day incubation with the solutions to be tested, at concentrations varying from 5% to 20% v/v in medium supplemented with 2% fetal calf serum devoid of endogenous estrogens, estrogenicity was assessed by cell counting; p-Estradiol was used as positive control. The statistical analysis of data was performed with two-way analysis of variance (ANOVA) with appliance and concentration as predictors. Differences were further investigated with the Tukey multiple comparison tests at the 0.05 level of significance. **RESULTS** No significant MCF-7 proliferation was induced by the three samples compared either to the eluents from as-received retainers or to the negative control. As expected, p-estradiol induced a potent stimulation of MCF-7 cell proliferation, while no effect was observed on MDA-MB-231 cells. **CONCLUSION** Under the conditions of this experiment eluents of as-received and retrieved Vivera® retainers did not seem to exhibit xenoestrogenic activity. **CLINICAL SIGNIFICANCE** Vivera® retainers can be used as part-time removable oral appliances following the manufacturer's instructions.

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In vitro assessment of cytotoxicity and estrogenicity of Vivera® retainers

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SHORT RUNNING TITLE: Cytotoxicity and estrogenicity of Vivera® retainers

STRUCTURED ABSTRACT

Aim: To investigate the cytotoxicity and estrogenicity of Vivera® retainers by assessing their biological behavioral effects as-received from the manufacturer and after retrieved from patients.

Materials & Methods: In this in vitro investigation six sets (maxillary and mandibular) of Viverra® retainers, three as-received and three retrieved after four weeks of use by patients of an orthodontic postgraduate clinic, were immersed in normal saline solution for 14 days following different modes of sterilization. The estrogenicity assays involved two cell lines, namely the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB-231. Following a six-day incubation with the solutions to be tested, at concentrations varying from 5% to 20% v/v in medium supplemented with 2% fetal calf serum devoid of endogenous estrogens, estrogenicity was assessed by cell counting; β -Estradiol was used as positive control. The statistical analysis of data was performed with 2-way ANOVA with appliance and concentration as predictors. Differences were further investigated with the Tukey multiple comparison test at the 0.05 level of significance.

Results: No significant MCF-7 proliferation was induced by the three samples compared either to the eluents from as-received retainers or to the negative control. As expected, β -estradiol induced a potent stimulation of MCF-7 cell proliferation, while no effect was observed on MDA-MB- 231 cells.

Conclusions: Under the conditions of this experiment eluents of as-received and retrieved Viverra® retainers did not seem to exhibit xenoestrogenic activity.

Clinical Significance: Viverra® retainers can be used as part-time removable oral appliances following manufacturer's instructions.

KEY WORDS

Cytotoxicity, estrogenicity, laboratory research Viverra® retainers

INTRODUCTION

Since post-orthodontic treatment changes occur due to the instability of the new occlusion produced by therapy but also due to growth, maturation and aging of the

dentition throughout life, appropriate retention protocols should be used for every individual. ^{1,2} Clear, removable thermoplastic retainers belong to this category and have become popular. A few years ago Align Technology Inc. (San Jose, CA) introduced a clear overlay device marketed as the Vivera[®] retainer. ³ This retainer with separate components fits over the upper and lower dental arches and follows the same 3D manufacturing process used in the fabrication of Invisalign[®] aligners. Polyurethane is the basic constituent polymeric component used in Invisalign[®] aligners material and is not entirely inert since the material is affected by heat, moisture, and prolonged contact with oral enzymes. ^{4,5} The new generation of Invisalign[®] aligner material is SmartTrack, a thermoplastic polyurethane with an integrated elastomer. ⁶

Some significant morphological differences have been found in the used Invisalign[®] aligners in relation to the new ones involving abrasion at the cusp tips, adsorption of integuments at stagnation sites, and localized calcification of the biofilm developed during intraoral use. ⁵ Similarly, their mechanical properties were adversely affected during intraoral aging. ⁷ Regarding leaching of biologically active substances, neither a traceable amount of substances in an ethanol aging solution after immersion of aligner specimens for two weeks at 23° C was detected ⁵ nor any cytotoxic and estrogenic activity of the device materials when tested in vitro were found. ⁴ Similarly Invisalign[®] aligners did not present any cytotoxic effect on human gingival fibroblasts, did not show any noticeable estrogenic effects when tested on MCF-7 breast cancer cell line, and no measurable Bisphenol-A (BPA) quantity release was traced in a trial of various orthodontic materials. ⁸ On the other hand, a relatively recent investigation found undesirable effects when epithelial cells were treated with eluates obtained from soaking Invisalign[®] plastic in saline solution. ⁹ This study was the first to report adverse

effect of contact with Invisalign® plastic on oral keratinocytes.

One crucial concern regarding the use of plastic-based materials is the leaching of chemical substances called xenoestrogens into the immediate environment surrounding the plastic. Those substances have the ability to produce a biological reaction comparable to that of estrogen hormones, which are capable of inducing estrogenic signals that modify gene expression.¹⁰⁻¹² One of the materials concerned is BPA, an important starting material for production of epoxy resins and polycarbonates, which is manufactured by acid catalyzed condensation of acetone and phenol.^{13,14} BPA exhibits great similarity in structure with 17 β -estradiol and may have similar effects.^{11,15} The accumulated level of BPA in the body may vary according to the developmental stage and gender of the subject. According to the United States Environmental Protection Agency reference dose and the Food and Drug Administration's acceptable daily intake dose, the presumed "safe" dosage is 50 μ g/kg/day of BPA.¹⁵⁻¹⁷ However, adverse effects have been documented with BPA doses below the above-mentioned daily level.

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Vivera® retainers seem to be produced by a similar material to the one used for Invisalign® aligners and may be characterized by similar properties. However, in contrast to Invisalign® aligners, which are usually used for maximum two weeks almost full-time, Vivera® retainers have been designed for prolonged use, normally on a part-time basis. This extended use could lead to degradation and possible deterioration of the material. A very recent report found statistically significant BPA levels in saliva in patients using vacuum-formed retainers.²²

Since no investigations have dealt with the cytotoxicity and estrogenicity of Vivera®

retainers until present such a study would be a valuable contribution to current knowledge.

The null hypothesis of this study was that Vivera® retainers, either as-received or after retrieval from patients, have no cytotoxic or estrogenic effect.

The aim of the present study was to investigate the cytotoxicity and estrogenicity of Vivera® retainers by assessing their biological behavioral effect as-received from the manufacturer and after retrieval from patients.

MATERIALS AND METHODS

The study sample consisted of six sets of Vivera® retainers, three as-received from the manufacturer and three retrieved from three consecutive patients of the Orthodontic Clinic, Hamdan Bin Mohammed College of Dental Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, UAE, who consented to be included in the study. With regard to the retrieved retainers, these were retrieved after four weeks of 12-hour a day use. Each set consisted of a maxillary and a mandibular appliance.

The evaluation of cytotoxicity and estrogenicity of all retainers took place in the Laboratory of Cell Proliferation and Ageing, Institute of Biosciences and Applications, National Centre for Scientific Research “Demokritos”, Athens, Greece. All retrieved retainers were divided in two equal parts randomly regardless of being upper or lower component. Each one was subjected to either mode of sterilization procedures, i.e. gamma-irradiation (IRR) or autoclaving (AUTOCL). The as-received retainers were divided into three equal parts randomly as well. Two parts were sterilized, with each part using one of the above-mentioned procedures, while the third part of as-received

retainers was not subjected to any sterilization mode, so as to test the effects of the sterilization procedure.

Following sterilization, all samples were immersed in sterile normal saline (NaCl 0.9% w/v) with each sample in different container and incubated for fourteen days at 37° C. Normal saline without any retainer was incubated under the same conditions in parallel, to be used as negative control. All retainers, which were following specific allocation and procedures of sterilization (Table 1), were aliquoted and kept at -20° C to maintain its integrity until further experimental use. Samples obtained from incubation of as-received/unsterilized retainers (i.e. samples 2 and 4) were considered to be identical (Table 1).

The estrogenicity assays involved two cell lines, i.e. the estrogen-sensitive MCF-7 and the estrogen-insensitive MDA-MB-231 (both from human breast adenocarcinoma), in order to exclude the possibility that a decreased proliferation of cells induced by the retainer eluent would mask a potential induction of proliferation due to estrogenicity.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, at 37° C, in 5% carbon dioxide, in a humidified incubator and regularly subcultured by using trypsin-citrate solution. To evaluate the estrogenicity of the samples, the cells were plated in 48-well flat-bottomed microwells (10,000 cells per well) in DMEM and 10% fetal calf serum. Twenty-four hours later, the medium was changed to phenol-free DMEM supplemented with 2% fetal calf serum pre-treated with dextran-coated charcoal, along with the solutions to be tested, at concentrations 5%, 10% and 20% v/v. β - Estradiol (βE_2) was used as positive control, and normal saline solution was used as negative control. After six days of incubation, with medium

renewal at day three, the cells were detached using trypsin-citrate solution and counted in a Z1 Beckman-Coulter counter. Assays were performed in triplicate, and the results were averaged.

The statistical analysis of data was performed with 2-way analysis of variance (ANOVA) with appliance and concentration as predictors. Differences were further investigated with the Tukey multiple comparison test at the 0.05 level of significance.

RESULTS

An initial experiment was performed using samples 1-3, corresponding to as-received retainers, to assess the effects of the two sterilization procedures while the third one served as a control. As shown in Graph 1, none of the samples, at any concentration tested, induced the proliferation of MCF-7 cells compared to the negative control, in contrast to the pronounced stimulation by all three β -estradiol concentrations (within the physiological limits) tested.

However, as shown in Figure 1, after gamma-irradiation, the appearance of the retainers was altered, acquiring a yellowish color reminiscent of the effect of ultraviolet light on plastic materials. Hence, it was considered that the sterilization through gamma-irradiation could potentially damage the plastic, and autoclaving was finally chosen as the preferred mode of sterilization.

Accordingly, samples 7, 10, and 12, corresponding to retrieved retainers from the three patients were evaluated in comparison to samples from as-received retainers (either autoclaved or not, i.e. samples 4 or 5).

As shown in Graph 2, no significant MCF-7 proliferation was induced by the samples 7,

10, and 12, compared either to the eluents from as-received retainers, i.e. 4 and 5, or to the negative control. As expected, β -estradiol induced a potent stimulation of MCF-7 cell proliferation, while no effect was observed on MDA-MB-231 cells.

Thus, the null hypothesis was not rejected meaning that Vivera[®] retainers either the as-received or after retrieved from patients demonstrated no cytotoxic or estrogenic action.

DISCUSSION

BPA's implication in the general use of aligners has not been conclusive at the cell culture or analytical level, with views such as their inert profile or BPA release supported by studies with different methodological approaches.²³

Since no other studies have assessed until present the cytotoxicity and estrogenicity of Vivera[®] retainers this investigation was designed to test them either in as-received or after use samples. The results failed to reveal any measurable adverse biological activity from either category, as-received or retrieved. A possible explanation could lie in the stability of material used for the fabrication of Vivera[®] retainers, which has not been stated as different from Invisalign[®] aligner material, with the latter being a polyurethane-derived one. This material is manufactured from polyether urethanes used as raw material and these polymers have short rigid portions (the aromatic rings and the ureas) joined by short flexible "hinges" (the diamine linker and the CH₂ group between the aromatic ring) and long very flexible portions (the polyether).²⁴ One should note that the chemical composition of this material as described does not contain the necessary ingredients to release BPA and induce its known adverse biological effects. However, it had been shown that some materials exhibit estrogenicity despite not containing BPA in

their composition.¹²

In this study the incubation period was for two weeks. In the present experimental set-up, the fact that during those two weeks, the normal saline immersion solution was left without being renewed should be taken into consideration. Accordingly, any effect likely to occur would be expected to be concentrated and amplified compared to the oral environment where saliva plays a role in diluting and renewing the medium as well as providing some protection effect.⁹

The estrogenicity of the eluent from the materials tested was measured using an established assay in protocol for estimating the proliferation of the estrogen-responsive MCF-7 cell line. These cells are known to express estrogen receptor- α , which is important for the proliferative effect of estrogens. It was proposed on account of its known intense proliferation upon exposure to very low levels of estrogens and, therefore, chosen for this sensitivity.²⁵ In addition, being of human origin the results of this study are more directly relevant to humans.

On the other hand, using an estrogen insensitive cell line, MDA-MB-231 to serve as a sham control was essential. This sham control aided to a more precise estimation of the estrogenicity of the tested materials as it excluded the possibility that the estrogenic proliferative effect could be masked by the cytostatic and/or cytotoxic action of the eluents.

17 β -estradiol is a natural hormone used in this study as a positive control, at a physiological concentration range.²⁶ This hormone is known to induce maximal biological effects at concentrations much lower than the levels at which all hormone receptors become saturated. Therefore, the lack of response to excessively high

concentrations of effectors could be misinterpreted as lack of effect.²⁶ On the other hand, even very low hormone concentrations (10^{-12} M) leading to only 1% occupation of receptors can induce MCF-7 cell proliferation.²⁶ Hence, the maximum concentration of the eluents from retainers used in this study (20% v/v) was considered adequate for estrogenicity assessment.

Including used retainers in the study was considered a strength, because it took into consideration the possibility that some material might react differently in the oral environment in terms of degradation and changes in physical and biological properties. The present study addressed this possibility by testing both conditions of the retainers namely as-received and retrieved from patients. With regard to the relatively short duration of the used retainers, which were retrieved after four weeks, research has shown an exponential release of BPA with high leaching in the first days thereafter followed by minimum.^{27,28}

All the retrieved retainers were subjected to one of the sterilization procedures, gamma irradiation or autoclaving, in order to eliminate any possibility of bacterial growth masking the results of the estrogenicity assay. Gamma-irradiation sterilized retainers, samples 6, 8, 9, and 11, were then excluded from the experiment as its appearance was distorted, getting a yellowish color.

This is the first study conducted for assessing the cytotoxic and estrogenic effect of this type of retainers. No other studies have been published dealing in a comparable way with the testing of the biological behavior of such material utilizing similar methodological processes apart from one report concerned only with as-received Invisalign® aligners and which produced similar results regarding the as-received

retainers. ⁴

The limitation of the current study lies in the results being based on in vitro assessment. However, in vivo testing cannot easily be performed due to ethical and practical difficulties involved, such as time required, difficulties in controlling the confounding variables, and frequent problems in interpreting the results. On the other hand, using cell cultures of human origin in this study is advantageous. ²⁹

CONCLUSIONS

Based on the results of this study, which was carried out to test the cytotoxic and estrogenic behaviour of both as-received and retrieved Viverra[®] retainers, it can be concluded that there is no significant estrogenic activity after the incubation of both groups of these retainers in normal saline for two weeks at body temperature.

CLINICAL SIGNIFICANCE

Viverra[®] retainers can be used as part-time removable oral appliances following manufacturer's instructions.

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This research was approved by the Research and Ethics Committee of the institution.

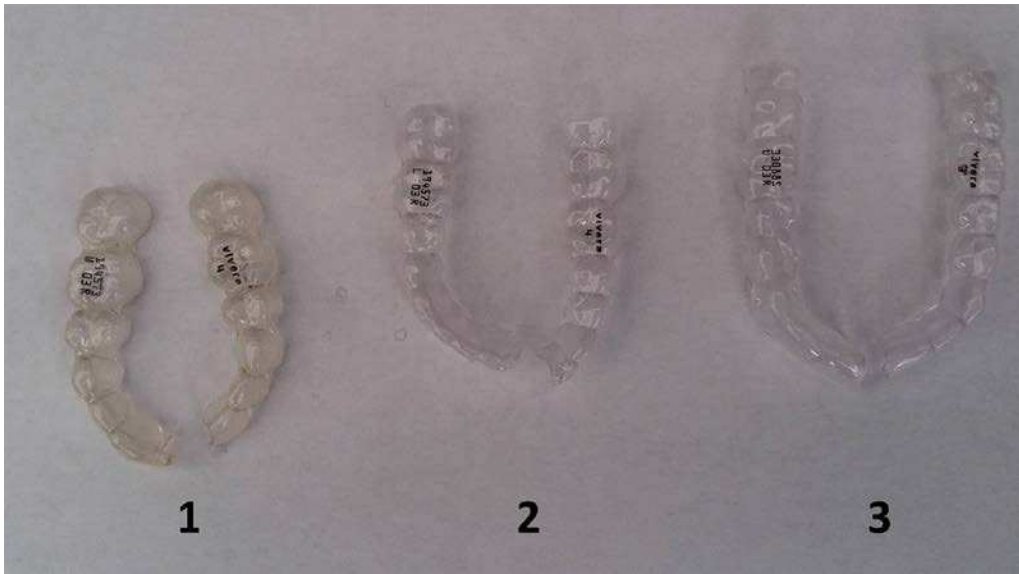
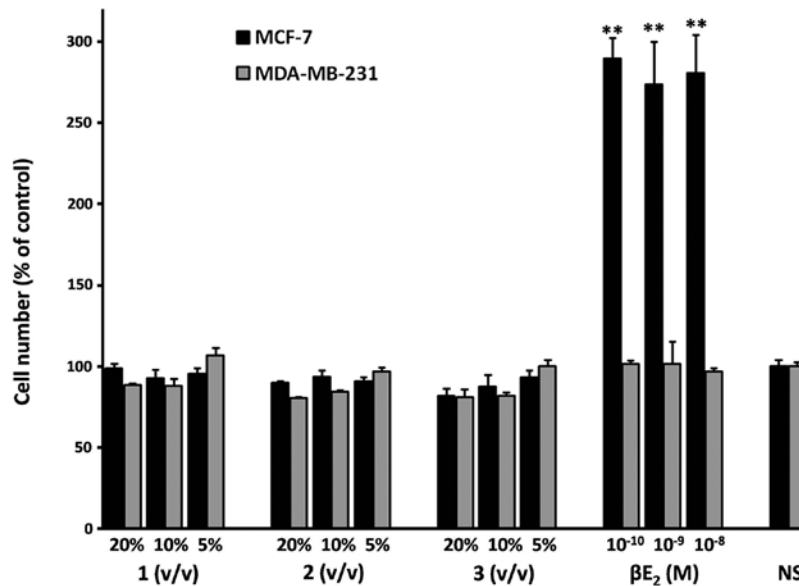


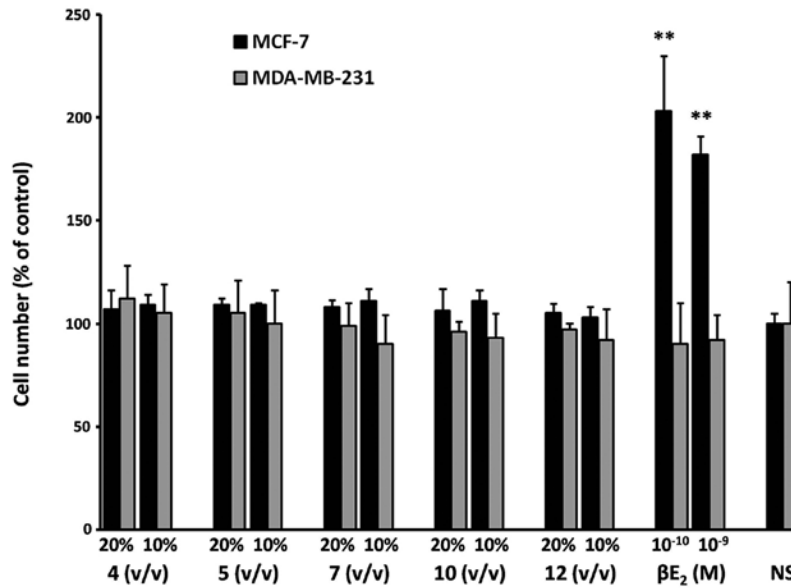
Figure 1. Appearance of the retainers after sterilization: Sterilized through gamma-irradiation (1); non-sterilized (2); and sterilized through autoclaving (3). After gamma-irradiation, the appearance of the retainers was altered, acquiring a yellowish color reminiscent of the effect of ultraviolet light on plastic materials. Hence, it was considered that the sterilization through gamma-irradiation could potentially damage the plastic, and autoclaving was finally chosen as the preferred mode of sterilization.

Table 1. Sample allocation and procedures of sterilization.

Sample No.	Patient code	Used retainer	Sterilization procedure
1	ALMFA014	No	IRR
2	ALMFA014	No	No
3	ALMAS000	No	AUTOCL
4	DOCTR000	No	No
5	DOCTR000	No	AUTOCL
6	DOCTR000	Yes	IRR
7	DOCTR000	Yes	AUTOCL
8	ALMAS000	No	IRR
9	ALMAS000	Yes	IRR
10	ALMAS000	Yes	AUTOCL
11	ALMFA014	Yes	IRR
12	ALMFA014	Yes	AUTOCL



Graph 1. Proliferation of MCF-7 vs. MDA-MB-231 cells in response to retainer eluent samples: effect of sterilization procedure using samples 1-3, corresponding to as-received retainers, to assess the effects of the two sterilization procedures while the third one served as a control. None of the samples, at any concentration tested, induced the proliferation of MCF-7 cells compared to the negative control, in contrast to the pronounced stimulation by all three β -estradiol concentrations (within the physiological limits) tested.



Graph 2. Proliferation of MCF-7 vs. MDA-MB-231 cells in response to retainer eluent samples (average from two experiments). No significant MCF-7 proliferation was induced by the samples 7, 10, and 12, compared either to the eluents from as-received retainers (samples 4 and 5) or to the negative control. As expected, β -estradiol induced a potent stimulation of MCF-7 cell proliferation, while no effect was observed on MDA-MB-231 cells.